PCT Applicant's Guide - Volume II - National Chapter ENS 3 4 Annex USIL page 1

'ASI3 Rec'd PCT/PTO 2 8 MAR 2001

F	TOTAT 1390 US DEPARTMENT O	F COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
Ψ	TRANSMITTAL LETT	447.001	
AP	2 8 200 DESIGNATED/ELE	CTED OFFICE (DO/EO/US)	U.S. APPLICATION NO. (If known, see 37 CFR 1.5
TIT.	2 0 2001 CONCERNING A FI	LING UNDER 35 U.S.C. 371	09/806368
ړ	INTERNATIONAL APPLICATION N		PRIORITY DATE CLAIMED
4	1B99/01621	October 4, 1999	October 9, 1998
		ROTEIN ANTAGONIST BASED OF	N THE MATURE PROTEIN
	APPLICANT(S) FOR DO/EO/US KATSUURA et al		
	Applicant herewith submits to the Unite	ed States Designated/Elected Office (DO/EO/US	the following items and other information:
	1. This is a FIRST submission of	items concerning a filing under 35 U.S.C. 371.	
	2. This is a SECOND or SUBSEC	QUENT submission of items concerning a filing	under 35 U.S.C. 371.
	This express request to begin not examination until the expiration.	ational examination procedures (35 U.S.C. 371(f. a of the applicable time limit set in 35 U.S.C. 371	)) at any time rather than delay (b) and PCT Articles 22 and 39(1).
	4. A proper Demand for Internation	nal Preliminary Examination was made by the 19th	month from the earliest claimed priority date.
	5. 🚰 A copy of the International Ap	plication as filed (35 U.S.C. 371(c)(2))	
		th (required only if not transmitted by the Interna	tional Bureau).in English
		by the International Bureau.	in a Office (PO/III)
		application was filed in the United States Received	
	<u>,                                    </u>	nal Application into English (35 U.S.C. 371(c)(2)	
	7. Amendments to the claims of t	he International Aplication under PCT Article 19 with (required only if not transmitted by the Inter	(35 U.S.C. 371(c)(3))
		d by the International Bureau.	dational Bulcauj.
		however, the time limit for making such amendr	nents has NOT expired.
		and will not be made.	
	8. A translation of the amendmen	its to the claims under PCT Article 19 (35 U.S.C.	. 371 (c)(3)).
	9. X Aπ oath or declaration of the i	nventor(s) (35 U.S.C. 371(c)(4)). Unexec	uted
	10. A translation of the annexes o (35 U.S.C. 371(c)(5)).	f the International Preliminary Examination Repo	ort under PCT Article 36
	Items 11. to 16. below concern de	ocument(s) or information included:	
	11. An Information Disclosure St		
	12. An assignment document for	recording. A separate cover sheet in compliance	with 37 CFR 3.28 and 3.31 is included.
	13. 🖾 A FIRST preliminary amendr	nent.	
	☐ A SECOND or SUBSEQUE	NT preliminary amendment	
	14. A substitute specification.	•	
	15. A change of power of attorne	-	
	16. X Other items or information:	International Preliminary Drawings (2 sheets); Secopy) part of application	equence Listing (paper
	·		
		•	
	ı		

U.S. APPLICATION NO. (if know	6 3 6 0		000 000 000 000 000 000 000 000 000 00			ATTORNEYS DOC 447.001	KET NUMBER
	<del>~~~~~~</del>		· 72/ UIUKI			LCULATIONS	PTO USE ON! Y
17. The follows  BASIC NATIONAL	_		:))-		<u> </u>		000 01101
Neither internation	• •				Ψ.	E00 00	
nor international se and International S	arch fee (37 CFR earch Report not	1.445(a)(2)) prepared by the	paid to USPTO the EPO or JPO	\$1070.00	\$ 	500.00	
International prelim USPTO but Intern	ninary examinatio ational Search Re	on fee (37 CFR port prepared	(1.482) not paid to by the EPO or JPO	\$930.00			
International prelin but international se	ninary examination earch fee (37 CFR	on fee (37 CFR 1.445(a)(2)) p	1.482) not paid to paid to USPTO	USPTO \$790.00			
International prelimbut all claims did n	ot satisfy provisi	ons of PCT Ar	ticle 33(1)-(4)	\$720.00			
International prelimand all claims satis	fied provisions of	f PCT Article :	33(1)-(4)	\$98.00	_	<del></del>	1
			IC FEE AMO		\$	500.00	
Surcharge of \$130.0 months from the ear	00 for furnishing t liest claimed pric	the oath or dec ority date (37 C	laration later than FR 1.492(e)).	20 30	s		
CLAIMS	NUMBER FII	ED NU	MBER EXTRA	RATE	\$		
Total claims	<del></del>	0 =		x \$22.00	S		
Independent claims	<u> </u>	=		x \$82.00	\$	<del> </del>	
MULTIPLE DEPEN	<del></del>		<del></del>	+ \$270.00	\$		<b>-</b>
D. Janes Company			BOVE CALCU		\$	500.00	<u> </u>
Reduction of 1/2 for must also be filed (1	r filing by small e Note 37 CFR 1.9,	ntity, if applic 1.27, 1.28).		+	s		
		<del></del>		JBTOTAL =	S	500.00	<b></b>
Processing fee of \$1 months from the ear	130.00 for furnish rliest claimed price	ority date (37 (	CFR 1.492(f)).		s		
			TOTAL NATIO		\$	500.00	
Fee for recording the accompanied by an	ne enclosed assign appropriate cove	nment (37 CFF r sheet (37 CF	k 1.21(h)). The ass R 3.28, 3.31). \$40	ignment must be .00 per property +	s		
		T	OTAL FEES E	NCLOSED =	2	500.00	
					Ап	nount to be refunded:	S
			···			charged:	2
a. X A check in	n the amount of	\$ 500.00	to cover t	he above fees is enclo	sed.		
	arge my Deposit			the amount of \$		to cover th	e above fees.
A duplica	te copy of this sho	eet is enclosed	-				
c. X The Commoverpaym	nissioner is hereb ent to Deposit Ac	by authorized to count No. 02	o charge any additi 2–2275 A dupli	onal fees which may l cate copy of this shee	be red t is e	quired, or credit and closed.	any
				1,495 has not been r ion to pending status		a petition to rev	ive (37 CFR
SEND ALL CORREST	PONDENCE TO:			~//	0	Alm_	
Bierman, N	Muserlian	and Luc	as	SIGNATI	URF	VIV	
600 Third			-	Charl		A. Muser	Jian
New York,	NY 10016			NAME 19,68		A. Muser	TTail
1							
			20311	REGISTI	KATIO	ON NUMBER	
		_ P/	TENT TRADEMARK OFFICE				

Our Ref.: 447.001

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

PCT/IB99/01621

• p

: PCT Date: April 10, 1999

Mieko KATSUURA et al

Serial No.:

:

Filed: Concurrently Herewith For: BONE...MATURE PROTEIN

:

600 Third Avenue New York, NY 10016

#### PRELIMINARY AMENDMENT

Assistant Commissioner for Patents Washington, D.C. 20231

Sir:

Page 1, before line 1, insert

--This application is a 371 of PCT/IB99/01621 filed October 4, 1999.--

## IN THE CLAIMS:

Claim 8 (amended) The mature protein according to claim 1, wherein said mature human MP52 is a dimer protein.

Claim 11 (amended) The mature protein according to claim 9, wherein said mature human BMP-2, mature human BMP-4, or mature human BMP-7 is a dimer protein.

Claim 12 (amended) An agent for therapy and/or prevention of ectopic ossification, containing a mature protein according to claim 1 as an effective ingredient showing an antagonistic activity agent against a bone morphogenetic protein.

Claim 13 (amended) An agent for therapy and/or prevention of metabolic diseases with calcification, containing a mature protein according to claim 1 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

### ADD THE FOLLOWING CLAIMS:

- --14. A method of treating ectopic ossification in warm-blooded animals comprising administering to warm-blooded animals in need thereof an amount of a mature protein sufficient to treat ectopic ossification.
- 15. A method of treating metabolic diseases with calcification in warm-blooded animals comprising administering to warm-blooded animals in need thereof an amount of a mature protein of claim 1 sufficient to treat said metabolic disease.--

#### REMARKS

The amendment is presented to insert reference to the PCT

application, to remove multiple dependency from the claims and to add method of use claims.

Respectfully submitted, BIERMAN, MUSERLIAN AND LUCAS

Charles A. Muserlian, #19,683

Attorney for Applicant(s) Tel. # (212) 661-8000

CAM:sd

Enclosures: Marked up Version of Claims

Return Receipt Postcard

24

### What is claimed is:

- A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.
- 10 2. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an oxidization reaction.
- The mature protein according to claim 2 in which four methionine residues are oxidized and having the amino acid
   sequence of SEQ ID N° 5.
  - 4. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an alkylation reaction.
  - 5. The mature protein according to claim 4 wherein the
- 20 alkylation reaction is S-carboxymethylation in which at least one methionine residue is S-carboxymethylated and having the amino acid sequence of SEQ ID N° 6.
  - 6. The mature protein according to claim 1, wherein the chemical modification for said tryptophane residue is an
- 25 allylsulphenylation reaction.
  - 7. The mature protein according to claim 6 in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID N° 7.  $_{A}$  .
  - 8. The mature protein according to claims 1 to 7, whereir
- 30 said mature human MP52 is a dimer protein.
  - 9. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID  $N^{\circ}$  2), mature
- 35 human BMP-4 (SEQ ID N° 3) or mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.

PCT/IB99/01621

25

- 10. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by replacing at least one amino acid residue of three hydrophobic amino acid residues, among said hydrophobic amino acid residues relating to a receptor binding site in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4), which are located in positions corresponding to those of methionine residues located in 30th, 71st, and 74th positions of the amino acid sequence of mature human MP52 (SEQ ID N° 1) with a hydrophilic amino acid residue or a polar amino acid residue.

  11. The mature protein according to claim 9 or 10, wherein said mature human BMP-2, mature human BMP-4, or mature human BMP-7 is a dimer protein.
- ossification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

  13. An agent for therapy and/or prevention of metabolic
- diseases with calcification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

JC03 Rec'd PCT/PTO 2 8 MAR 2001

WO 00/21998

5

PCT/IB99/01621

7

MATURE PROTEIN HAVING ANTAGONISTIC ACTIVITY AGAINST BONE MORPHOGENETIC PROTEIN

#### BACKGROUND OF THE INVENTION

(1) Field of the Invention

This invention relates to a mature bone morphogenetic protein of which some hydrophobic amino acid residues are exchanged to a hydrophilic or a polar amino acid residue by chemical modification or genetic engineering technology. The nature proteins of this invention show an antagonistic activity against bone morphogenetic proteins, and are useful for medicinal agents to suppress symptoms of ectopic osteogenesis and ectopic calcification or metabolic bone diseases with calcification such as neurotic osteosis, ectopic ossification caused by stress of operation, traumatic myositis ossificans, ossification by defect of oxygen supply, osteogenic tumor, ossification of the posterior longitudinal ligament, and arterial sclerosis.

- (2) Description of the Related Art
- A bone morphogenetic protein (hereinafter called BMP) is a protein having a bone morphogenetic activity in decalcified bone tissue. Although the isolation of BMP had been worked on energetically since the 1970s, it was quite difficult to isolate as a single protein. Gene cloning of BMP as expected
- was performed by Wozney in 1989 by molecular biological technology, using the amino acid sequences derived from unknown peptides which were separated by treating the fraction having bone morphogenetic activities with an enzyme. The gene was immediately introduced to the animal cultured
- 30 cells, and the activity of the protein expressed was measured in vivo, and BMP activity in the protein was practically proved (Wang, E. A. et al., (1990) Proc. Natl. Acad. Sci. USA, vol. 87, p. 2220-2224). Continuing cloning a protein with bone morphogenetic activities utilizing homology,
- 35 several numbers of the proteins with bone morphogenetic activities in a similar structure have been isolated so far. Those proteins all belong to TGF (transforming growth factor)- $\beta$  superfamily and are proved to have the activity to

2

cause ectopic ossification in vivo, basically. Ossification caused by BMP is said to be internal cartilaginous and it seems to reproduce the formation of long bone at an embryonal stage. Therefore, BMP itself can be used as a medicinal agent for the treatment to compensate the bone deficit.

On the other hand, since BMP genes were disclosed and the specific antibodies against BMPs were prepared, BMPs were also expressed at the site of ectopic calcification, which have not had any medical treatment so far, and there seem 10 some possibilities of the relationship between BMPs and those diseases. For example, it becomes recently evident that BMP exists or is included in diseases such as neurotic osteosis. ectopic ossification caused by stress of operation, traumatic myositis ossificans, ossification by defect of oxygen supply, 15 osteogenic tumor, specified as refractory diseases such as ossification of the posterior longitudinal ligament (OPLL) (Spine, 17-3S, S33, 1992) and calcification part of arterial sclerosis (J. Clin. Invest., vol. 91, p. 1800, 1993). addition, the major symptoms of pseudomalignant heterotopic 20 ossification (PHO), pseudomalignant osseous tumor and myosistis ossificans circumscripta are ache and the

myosistis ossificans circumscripta are ache and the appearance of the hard tissue mass in the muscle. Though the causes of these diseases are still unknown in detail, BMP seems to have a relationship with the appearance of hard

25 tissue in the muscle of the patients. It is considered that BMP exists in the tissue in which BMP does not exist naturally, acts on autocrine, and forms bone like hard tissue. There is no effective treatment for OPLL by now. When the compressive neural symptom is critical, an excision 30 is operated. However the prognosis is not so good. There is no treatment for calcification of artery, neither.

It seems that suppression of BMP existence may be one of the major treatments for these diseases. Another treatment, such as administration of BMP antagonists, also seems

35 effective. BMP receptors, neutralized antibodies against BMP and the BMP peptides corresponding to the binding site, a BMP with chemically modified specific amino acid residue are thought to have BMP antagonist-like activity.

3

Many studies have been so far carried out concerning a relationship between the structure and the activity of EMPs and it is speculated that some sites of mature BMPs relate to the receptor binding. It is known that a peptide synthesized based on these studies works as an antagonist against BMPs (JP patent application, Hei 7 ('95) - 200175).

SUMMARY OF THE INVENTION

Other than the synthetic peptides, many kinds of methods are desired to exist for therapy of various diseases of bones and cartilages such as hyperplasia. The purpose of this invention is to provide a new BMP antagonist protein as an effective therapeutic agent for above bone-related diseases in which a specific amino acid residue is chemically modified or replaced by genetic engineering technology.

#### 15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a comparison of ALPase inducing activities by methionine alkylated mature human MP52s and that of unmodified mature human MP52 in MC3T3-El cells, which were separated by the difference in the retention time on the reverse phase HPLC. In the figure, the solid line with solid circles represents unmodified mature human MP52, the solid line with solid squares represents one-methionine alkylated mature human MP52, the broken line with solid triangles represents two-methionine alkylated mature human MP52, the solid line with open circles represents three-methionine alkylated mature human MP52, the dotted line with open triangles represents four-methionine alkylated mature human MP52, respectively. The solid circles shown in of the Y-axis represents ALPase activity in cells without treated by any reagent.

Fig. 2 shows the antagonistic activity of methionine oxidized mature human MP52 and tryptophane allylsulphenylated mature human MP52 in different cell lines. (A) represents their antagonistic activities against mature rh-BMP-2 in C3H10T ½ cells and (B) shows their antagonistic activities against mature human MP52 in MC3T3-El cells, respectively. In both figures, the solid line with solid circles represents tryptophane allylsulphenylated mature human MP52 and the

\*\*\*

WO 00/21998 PCT/IB99/01621

4

dotted line with open circles represents methionine oxidized mature human MP52, respectively. In (A), the solid square shows ALPase activity induced by 300 ng/ml of mature rh-BMP-2 alone in C3H10T ½ cell. In (B) the solid square shows ALPase activity induced by 600 ng/ml of unmodified mature human MP52 alone in MC3T3-El cells. Open squares represent the activity in the experiments without any reagent.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue by genetic engineering technology.

When at least one residue of said methionine is converted, chemical modification for said methionine residue 20 is an oxidization reaction or an alkylation reaction.

When the chemical modification of said methionine residue is an oxidization reaction, among the preferred mature proteins of the invention is that in which four methionine residues are oxidized and having the amino acid sequence of SEQ ID N° 5.

When the chemical modification of said methionine residue is an alkylation reaction, among the preferred mature proteins of the invention is that in which at least one methionine residue is S-carboxymethylated and having the 30 amino acid sequence of SEQ ID N° 6.

When at least one residue of said tryptophane is converted, chemical modification for said tryptophane residue is an allylsulphenylation reaction.

Among the preferred mature proteins of the invention is 35 that in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID N° 7.

The present invention relates to mature proteins of the invention as defined above having an antagonist activity

5

against bone morphogenetic proteins wherein said mature human MP52 is a dimer protein.

Further, the present invention relates to a mature protein having an antagonistic activity against bone

5 morphogenetic proteins, obtained by converting at least one residue of tryptophane residues existing in the amino acid sequence of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing

10 said residues with a hydrophilic amino acid residue or a polar amino acid residue by genetic engineering technology.

Furthermore, the present invention relates to a mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by replacing at least one amino acid residue of three hydrophobic amino acid residues, among said hydrophobic amino acid residues relating to a receptor binding site in the amino acid sequence of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4), which are located in positions corresponding to those of methionine residues located in 30th, 71st, and 74th positions of the amino acid sequence of mature human MP52 (SEQ ID N° 1), with a hydrophilic amino acid residue or a polar amino acid residue by genetic engineering technology.

The present invention relates to mature proteins of the invention as defined above having an antagonist activity against bone morphogenetic proteins, wherein said mature human BMP-2, mature human BMP-4 or mature human BMP-7 is a dimer protein.

The present invention, further, relates to an agent for therapy and/or prevention of ectopic ossification, containing a mature protein of the invention defined above as an effective ingredient showing an antagonistic activity against bone morphogenetic proteins.

Furthermore, the present invention relates to an agent for therapy and/or prevention of metabolic diseases with calcification, containing a mature protein of the invention defined above as an effective ingredient showing an

6

antagonistic activity against bone morphogenetic proteins.

The proteins belonging to the TGF-  $\beta$  superfamily are each presumed from its gene structure to be synthesized as a precursor in cells and to be formed into a mature (active type) peptide homodimer after various processings. The active type of mature human TGF- $\beta$ l is known to be a dimer of the peptide of COOH-terminal 112 residues (Nature, 316, 701-705, 1985). The term "mature BMP" as used herein means a BMP which is substantially composed of an amino acid sequence 10 homologous with COOH-terminal 112 amino acid residues of active type of human TGF- $\beta$ l. The proteins belonging to the TGF- $\beta$  superfamily in the present invention are exemplified as BMP-2, BMP-4, BMP-7, human MP52 and so on.

The inventors attempted a chemical modification and a replacement with an amino acid for human MP52 that belongs to  $TGF-\beta$  superfamily and particularly that is a member of BMP family, with a purpose of preparing a chemically modified protein strongly acting as a BMP antagonist through binding to BMP binding site on the receptor.

It is assumed that the activated type of human MP52 is a dimer form and that the receptor binding site of mature MP52 is an exposed part on the outside of the protein molecule. Thus, the receptor binding site is speculated that it is composed of two vicinal peptide regions (the amino acid positions from the 20th Arg to the 38th Ala and from 60th Glu to 77th Glu in SEQ ID N° 1 of the Sequence Listing) in formation of a dimer of human MP52.

We prepared a protein of which two kinds of amino acid residues were chemically modified because it is known that the content of tryptophane and methionine is generally low in amino acid composition of the protein allowing selective chemical modification, and from the assumed three-dimensional structure of human MP52 that three (30th, 71st, and 74th positions) of four methionine residues in the monomer of mature human MP52 exist in the assumed receptor binding site and that both (32nd and 35th) of two tryptophane residues in the monomer of mature human MP52 exist in the assumed receptor binding site of mature MP52.

7

Chemical modification of this invention means that the side chains of methionine or tryptophane are modified in a degree enough to make the protein inactive with keeping a binding ability to the receptor. For the chemical 5 modification, a conventional method in studies on protein chemistry is applied. The method is described in "Protein Chemistry IV, " pp 6-118, 1977, Jap. Soc. Biochem. Methionine is a hydrophobic amino acid and can be modified into a hydrophilic residue by chemical modification. Chemical 10 modifications in increasing hydrophilicity are exemplified by oxidization of methionine to make methionine sulfoxide or methionine sulfon using an oxidant and by alkylation to make S-carboxymethylmethionine using  $\alpha$ -halogenoacetic acid. oxidization of methionine are performed by hydrogen peroxide, 15 performic acid, periodic acid, N-chlorosuccinimide, and Nbromosuccinimide. Besides, for  $\alpha$ -halogenoacetic acid, monoiodoacetic acid and monoiodoacetamide can be used.

Chemical modification to indole ring of tryptophane gives a subtle change of environment and structure nearby it.

20 Suitable examples are allylsulphenylation of the second position of indole nucleus by p- or o-nitrophenyl-sulphenylchloride, 2-nitro-4-carboxyphenylsulphenylchloride, 2,4-dinitrophenylsulphenylchloride, or 2-hydroxy-5-nitrobenzylation of the 3rd position of indole nucleus by 2-hydroxy-5-nitrobenzylbromide, dimethyl(2-hydroxy-5-nitrobenzylbromide) sulfonylbromide.

The present invention in particular relates to a mature protein of which one to 4 methionine residues or 1 to 2 tryptophane residues are chemically modified in mature human 30 MP52 amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing.

The protein shown in SEQ ID N° 1 of the Sequence Listing can be produced by the method described in International Patent Application WO 96/33215. Since 3 out of 4 methionine residues exist in the receptor binding site of mature human MP52 produced by said method, and all 2 tryptophane residues exist there, and whose amino acids are all hydrophobic, it is assumed that these hydrophobic amino acids play an important

÷.

WO 00/21998 PCT/IB99/01621

8

role in bone morphogenetic activity.

Mature human MP52 of the present invention includes not only the mature protein with the amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing but also Ala or Arg-Ala binding to the N-terminus of the amino acid sequence of said mature protein as disclosed respectively in international patent application WO 95/04819 and WO 97/06254.

Comparing the amino acid sequences of the mature proteins of other BMPs such as human BMP-2 (SEQ ID N° 2), 10 human BMP-4 (SEQ ID N° 3), human BMP-7 (SEQ ID N° 4) and so on with that of mature human MP52 protein, it is found that in those bone morphogenetic proteins the amino acids corresponding to the positions of methionine residues of mature human MP52 are all methionine residues or hydrophobic 15 amino acids.

In concrete, the receptor binding site of mature human BMP-2 is estimated to be the peptides with the amino acid sequence from 16th Arg to 34th Ala and from 56th Asn to 73th Lys shown in SEQ ID N° 2 of the Sequence Listing (Science 242, 1528-1534, 1988). The amino acids of mature human BMP-2, which corresponds to methionines' position of the mature human MP52 (30th, 71st and 74th), are replaced with 26th Val, 67th Val and 70th Val.

The receptor binding site of mature human BMP-4 is
estimated to be the peptides with the amino acid sequence
from 18th Arg to 36th Ala and from 58th Asn to 75th Ser shown
in SEQ ID N° 3 of the Sequence Listing (DNA Seq. 5 (5), 272275, 1995). The amino acids of mature human BMP-4, which
corresponds to methionines' position of mature human MP52

30 (30th, 71st and 74th), are replaced with 28th Val, 69th Val
and 72rd Val.

The receptor binding site of mature human BMP-7 is estimated to be the peptides with the amino acid sequence from 40th Lys to 58th Ala and from 80th Asn to 97th Glu shown in SEQ ID N° 4 of the Sequence Listing (Proc. Natl. Acad. Sci. U.S.A. 93(2), 878-883, 1996). The amino acids of mature human BMP-7, which corresponds to methionines' position of mature human MP52 (30th, 71st and 74th), are replaced with

9

50th Leu, 91st Val and 94th Ile.

It is difficult to carry out a chemical modification selectively on the hydrophobic amino acids of which positions correspond to those of methionine of mature human MP52.

5 However, it is possible to replace hydrophobic amino acids with hydrophilic or polar amino acids by genetic engineering technology.

Since the tryptophane residues are conserved in all BMPs described above, it is possible to replace hydrophobic amino acids with hydrophilic or polar amino acids by chemical modification method or genetic engineering technology. The tryptophane residues also play an important role in making a change in a three-dimensional structure in chemical modification and genetic engineering technology.

15 Thus, the present invention relates to mature human MP52, in particular, which is obtained by converting one to 4 methionine residues or one to 2 tryptophane residues existing in the amino acid sequence shown in SEQ ID N° 1 of the Sequence Listing to hydrophilic residues or polar amino acid 20 residues by recombinant engineering technology. In the present invention hydrophilic amino acids and polar amino acids are aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on. In the present invention, when methionine residues or tryptophane residues 25 are converted to other hydrophilic or polar amino acids, the protein of the present invention can be obtained by transforming a recombinant DNA into E. coli which are made by replacing the codons corresponding to methionines at 30th, 71st, 74th and 111th positions or the codons corresponding to 30 tryptophanes at 32nd and 35th positions shown in SEQ ID N° 1 replaced with the codons of aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine and so on or by making.

Further, the present invention relates to any mature 35 proteins having the amino acid sequence shown in SEQ ID Nos 2 to 4, of which one to two tryptophane residues are subjected to chemical modification.

The present invention furthermore relates to any mature

10

proteins having the amino acid sequence shown in SEQ ID Nos 2 to 4, in which hydrophobic amino acids consisting of the receptor binding site, 1 to 3 hydrophobic amino acids corresponding to the positions of 30th, 71st and 74th methionine residues or 1 to 2 tryptophane residues are replaced with hydrophilic amino acids or polar amino acids. In the present invention, hydrophobic amino acids are methionine, valine, leucine, isoleucine or tryptophane.

The present invention relates to the mature protein

10 having the amino acid sequence shown in SEQ ID N° 5 of the

Sequence Listing, in which 4 methionine residues of the

methionine residues are oxidized. In detail, for the

oxidization of methionine residue, hydrogen peroxide is added

at a final concentration of 0.014% to 2 mg/ml of the mature

15 protein and the reaction is carried out for more than 15

hours at room temperature and then the mature protein of

which methionine residues are in methionine sulfoxide can be

obtained.

The present invention relates to the mature protein

20 having the amino acid sequence shown in SEQ ID N° 6 of the
Sequence Listing, in which 1 to 4 methionine residues of the
methionine residues are alkylated. In detail, monoiodoacetic
acid is added at a concentration of higher than methionine
residues by 50 to 100 at the molar ratio to 2 mg/ml of the

25 mature protein and the reaction is carried out for more than
15 hours at room temperature and then the mature protein of
which methionine residues are S-carboxymethylated can be
obtained.

The present invention relates to the mature protein

30 having the amino acid sequence shown in SEQ ID N° 7 of the
Sequence Listing, in which 2 tryptophane residues of the
trypophane residues are allylsulphenylated. In detail, 20
equivalents of p-nitrophenylsulphenyl chloride in 100% acetic
acid is added to 2 mg/ml of the mature protein and the

35 reaction is carried out for 1 hour at room temperature and
then the mature protein of which tryptophane residues are
allylsulphenylated can be obtained.

The present invention relates to the mature protein

11

having an amino acid sequence shown in SEQ ID  $N^{\circ}$  2 of the Sequence Listing, in which 1 to 2 tryptophane residues at 28th and 31st are allylsulphenylated.

The present invention relates to the mature protein

5 having an amino acid sequence shown in SEQ ID N° 2 of the
Sequence Listing, in which one or all of 26th, 67th and 70th
valine residues or one or both of 28th and 31st tryptophane
residues are replaced with aspartic acid, glutamic acid,
lysine, arginine, histidine, serine, threonine and so on.

The present invention relates to the mature protein having an amino acid sequence shown in SEQ ID N° 3 of the Sequence Listing, in which one or both of 30st and 33th tryptophane residues are allylsulphenylated.

10

20

13

The present invention relates to the mature protein

15 having an amino acid sequence shown in SEQ ID N° 3 of the

Sequence Listing, in which one or all of 28th, 69th and 72rd

valine residues or one or both of 30st and 33th tryptophane

residues are replaced with aspartic acid, glutamic acid,

lysine, arginine, histidine, serine, threonine and so on.

The present invention relates to the mature protein having an amino acid sequence shown in SEQ ID N° 4 of the Sequence Listing, in which one or both of 52nd and 55th tryptophane residues are allylsulphenylated.

The present invention relates to the mature protein

25 having an amino acid sequence shown in SEQ ID N° 4 of the

Sequence Listing, in which one or all of 50th leucine, 91st

valine, 94th isoleucine, 131th methionine or one or both of

52nd and 55th tryptophane residues are replaced with aspartic

acid, glutamic acid, lysine, arginine, histidine, serine,

30 threonine and so on.

The BMP antagonist-like activity of the protein modified chemically or replaced with other amino acids in the present invention can be proved by measuring alkaline phosphatase (ALPase) activity as a marker of biological activity by adding in a culture medium of a murine carvarial clonal cell line (MC3T3-E1 cell) having osteoblastic property which Kodama et al. established (Kodama, H. et al. (1981) Jpn. J. Oral Biol., vol. 23, p.899). ALPase is often used as a

12

marker enzyme for differentiation/maturation in osteoblasts and cartilage cells (Pfeilschifter, J., et al., Endocrinology (1987), vol. 121, p 212-218; Rodan, G. A., et al., Calcium regulating hormones and bone metabolism, Elsevier Science Publishers B.V., (1992), p 183-196).

The mature protein obtained by examples 1 to 3 in the present invention inhibited dose-dependently the increase of ALPase activities which were induced by recombinant mature human BMP-2 (rh-BMP-2) or mature human MP52 in MC3T3-E1 cell line and C3H10T % cell line having osteoblastic property. The result indicated that the protein in the present invention could inhibit not only the activity of human MP52 but also the activities of other BMPs.

The present invention relates to an agent for therapy and/or prevention of ectopic ossification, containing any protein described above as an effective ingredient.

Furthermore, the present invention relates to an agent for therapy and/or prevention of metabolic diseases with calcification, containing any mature protein described above 20 as an effective ingredient.

The protein of the present invention is effective in therapy as an agent of suppressing worsening condition of OPLL and arterial sclerosis, treating tumor in bone and cartilage in which BMP is expressed and treating other

25 metabolic bone diseases. It can suppress worsening condition by lowering the function of osteoblasts by using over cycle of metabolic bone such as Paget's disease. It can also be useful as a reagent for screening or evaluating systems of medicinal agents which compete with BMP and the receptor binding.

The administrating method can be exemplified as intravenous and intramuscular administrations. Not only a standard intravenous injection but also intravenous drip infusion can be applied to intravenous administration.

For example, it can be prepared as a powder preparation for injection. In this case, one or more than two kinds of water soluble excipients such as mannitol, sugar, lactose, maltose, glucose and fructose may be added to the agent and

13

dissolved in water. And after putting the mixture into vials or ampoules, they are freeze-dried and then sealed to be an injection preparation.

Although the adult clinical administration dosage for a day may vary from and also depend on administrating methods, ages, weigh, conditions of patients and so on, it is usually 0.01 - 5 mg of the protein.

This invention is described in detail by examples written below. However, this invention is not restricted to 10 these examples.

#### EXAMPLES

Example 1 Preparation of mature human MP52 of which methionine residue is oxidized

(1) Oxidization of methionine residue of mature human MP52 by 15 hydrogen peroxide (conversion to methionine sulfoxide)

Hydrogen peroxide was added to mature human MP52 (2 mg/ml concentration) dissolved in 2 mM EDTA - 10 mM hydrochloric acid at the final concentration of 0.014% and the solution was reacted at room temperature for more than 20 15 hours.

(2) Separation of methionine oxidized mature human MP52

Mature human MP52 having the amino acid sequence shown in SEQ ID N° 1 (hereafter, unmodified mature human MP52) and methionine oxidized mature human MP52 having the amino acid sequence of SEQ ID N° 5 were separated by using reverse phase HPLC on the basis of a difference between retention times of both proteins. Oxidization increased a hydrophilic property of the proteins and retention time methionine oxidized mature human MP52 on the reverse phase HPLC becomes faster than that of unmodified mature human MP52. The conditions of separation are as follows. For column, Nucleosil 5-C18-300 column (4.6 mm I.D. X 150 mm, GL Science Corp.) was used at the flow rate of 1.3 ml per minute at 45°C; absorbance at 214 nm and 280 nm were measured to detect peaks. For solvent,

35 water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of proteins were performed with a linear gradient of solution B from 25% to 45% for 80 minutes using an HPLC pump, HP1050

Sin

WO 00/21998 PCT/IB99/01621

14

(Hewlett Packard).

Under this condition, unmodified mature human MP52 is eluted at around 51 minutes, whereas methionine oxidized mature human MP52 was eluted at around 47 minutes. Thus, 5 both were easily separated.

(3) Determination of the oxidization of methionine residue

The oxidization of methionine residue was detected by
comparing elution patterns of the reverse phase column
chromatography of trypsin digested fragments of unmodified

10 mature human MP52 and methionine oxidized mature human MP52.

Unmodified mature human MP52 has seven cysteine residues as shown in SEQ ID N° 1 of the Sequence Listing. Six out of seven cysteine residues form three intramolecular disulfide bonds and the remaining one cysteine forms a dimer. For complete enzymatic digestion, reducing disulfide bond and blocking SH group are required to inhibit rebinding. For this purpose, dithiothreitol was used to reduce the disulfide bond and the cysteine residue was alkylated (S-carboxymethylation) before trypsin digestion.

First, lyophilized unmodified mature human MP52 and methionine oxidized mature human MP52 were dissolved in 8 M urea - 0.2 M ammonium bicarbonate - 2 mM EDTA (pH 8.5) at the final concentration of 1 mg/ml, and 50-fold molar excess of dithiothreitol (DTT) to the cysteine residue was added and

- reacted at 50°C for 30 minutes. To this solution, 250-fold molar excess of monoiodoacetamide to the cysteine residue was added and reacted for 30 minutes with shading to yield S-carboxymethylated unmodified mature human MP52 and S-carboxymethylated methionine oxidized mature human MP52.
- 30 Digestion of these proteins was performed by adding trypsin. After four-fold dilution of this solution with water to make the final urea concentration of 2 M, at the weight ratio of 1/50 to the proteins at 37°C for 18 hours, the trypsin digestion was applied to the reverse phase HPLC column to
- 35 separate all fragments. The conditions of separation are as follows. Nucleosil 5-Cl8-300 column (4.6 mm I.D. X 150 mm, GL Science Corp.) was used for separation at the flow rate of 1.3 ml per minute at 45°C; absorbance at 214 nm was measured

15

to detect peaks. For solvent, water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of peptides was performed by a linear gradient of solution B from 0% to 45% for 90 minutes after 5 keeping 0% for initial five minutes, using HPLC pump, HP1050 (Hewlett Packard).

Subsequently, amino acid composition analysis was carried out to determine the positions of the digested peptide on the primary structure of reduced alkylated

10 unmodified mature human MP52 and reduced alkylated methionine oxidized mature human MP52. The operation of the amino acid composition analysis was mainly based on Zoku Seikagaku Zikken Kouza (Tokyo Kagaku Doujin), Vol. 2, Protein Chemistry (I), Section 4. Brief description is given below.

- 15 Hydrolysis was performed in a vapor of 6 N HCl containing 0.1% phenol at 110°C for 21 hours by using PICO. TAG. WORK STATION (Waters). Following this step, the amino acid composition analysis was carried out by PTC method by using Amino acid standard H (Pierce) as a standard amino acid.
- 20 PTC-amino acids were separated by reverse phase HPLC using HPLC pump (model 510; Waters), a Wakopak WS-PTC (4.0 mm I.D. X 200 mm; Wako Pure Chemicals), and solvents of PTC amino acids eluent A and PTC amino acids eluent B (both Wako Pure Chemicals).
- 25 The HPLC retention times of the identified trypsin peptides on the primary structures of unmodified and oxidized mature MP52 were compared each other. The trypsin fragments of mature human MP52 containing methionine residues were three kinds corresponding to: position from 29th to 56th (29-30 56; containing 30th methionine) shown in SEQ ID N° 1, position from 57th to 88th (57-88; containing 71st, and 74th methionines) in SEQ ID N° 1, and position from 107th to 119th (107-119; including 11th methionine) in SEQ ID N° 1. The elution time of respective fragments derived from the reduced alkylated unmodified mature human MP52 were around 84 min, 62 min, and 36 min in order. In comparison, the fragments derived from the reduced alkylated methionine oxidized mature human MP52 (SEQ ID N° 5) were around 80 min, 48 min, and 31

16

min in order showing the earlier elution than that of the fragments derived from the reduced alkylated unmodified mature human MP52.

On the other hand, there was no difference for other 5 fragments which do not contain methionine. As a result, the specific reaction of oxidization to the methionine residues was determined.

Example 2 Preparation of mature human MP52 of which methionine residue was S-carboxymethylated

10 (1) Alkylation of methionine residue of mature human MP52 by monoiodoacetic acid

Though the highest reactivity of alkylation using monoiodoacetic acid is observed in SH residues of cysteine residue, the alkylation reaction almost selectively occurs in methionine residue in an acidic condition, because all the cysteine residues of unmodified mature human MP52 form disulfide bonds as described before. Therefore, the operation was conducted as follows. Monoiodoacetic acid (molar ratio; 50-100 times higher than moles of methionine residue) was added to unmodified mature human MP52 with a concentration of 2 mg/ml dissolved in 10 mM hydrochloric acid and incubated at room temperature for 3-18 hours.

- (2) Separation of mature human MP52 of which methionine residue was alkylated
- Unmodified mature human MP52 and methionine alkylated mature human MP52 having the amino acid sequence of SEQ ID N° 6 were separated on the basis of a difference in retention times between both proteins by using reverse phase HPLC. The conditions of separation (a column employed, a flow rate,
- 30 wavelengths for detection, and a pump for HPLC) were the same as those of Example 1 (2). For solvent, water containing 0.05% TFA as solution A and acetonitrile containing 0.05% TFA as solution B were used. Elution of the proteins were performed by a linear gradient of solution B from 30% to 45% 35 for 60 minutes after five minutes of an initial condition.

Under this condition, unmodified mature human MP52 is eluted as one peak around 38 minutes and, in contrast to this, alkylated mature human MP52 showed four peaks (elution

17

times: 36 min, 34 min, 32 min, and 29 min) other than one peak around 38 minutes which is the elution time of unmodified mature human MP52. Elution time becomes earlier in accordance with proceeding of the reaction, namely,

5 depending on the increasing number of alkylated methionines. These peaks were collected to identify these proteins by the amino acid composition analysis similar to Example 1 (3). In the amino acid composition analysis, alkylated methionines are detected as a separate peak from unmodified methionines,

10 because alkylated methionines are not decomposed by hydrolysis. Namely, the progress of the reaction can be determined by a decrease in methionine peaks.

By the separation experiment, it was known that the elution peak at 29-min was that of all four methionines

15 alkylated mature MP52, the elution peak at 32-min was that of three methionines alkylated one, the elution peak at 34-min was that of two methionines alkylated one, the elution peak at 36-min was one methionine alkylated one. In addition, the elution peak at around 38-min was known as unalkylated one.

Amino acid compositions of these peaks were good agreement with those of theoretical values of mature human MP52 except for the value of methionine. Consequently, it is confirmed that the alkylation reaction to methionine residue occurs specifically and the separated peaks show progressing stages of the alkylation reaction differently.

(3) Determination of the alkylated methionine residues

The alkylated methionine residue was determined by
comparing elution patterns of trypsin digested fragments of
unmodified mature human MP52 and methionine alkylated mature
30 human MP52 on the reverse phase HPLC.

Similar to Example 1 (3), cysteine residues were blocked by alkylation before trypsin digestion after reduction of disulfide bonds by using dithiothreitol. Since S-carboxymethylation was performed using monoiodoacetic acid for the alkylation of methionine; alkylation of cysteine was performed by using 4-vinyl pyridine as an alkylation reagent for monoiodoacetic acid, which was not similar to Example 1. Alkylation reaction takes place almost selectively in reduced

18

cysteine at pH 8.5, and hardly in methionine. Thus, the reason of employing different alkylation is to detect side reaction in methionine.

First, lyophilized unmodified mature human MP52 and 5 methionine alkylated mature human MP52 were dissolved in a solution of 6 M quanidine - HCl - 0.4 M Tris-HCl buffer (pH 8.5) at the final concentration of 1 mg/ml, and reduced with 50-fold excess molar of dithiothreitol (DTT) to the cysteine residues at 50°C for 30 minutes. S-pyridyl-ethylation was 10 performed by adding 250-fold excess molar of 4-vinyl pyridine to the cysteine residues and incubated for 30 minutes with shading. The solution was applied on a desalting column (PD-10; Pharmacia) equilibrated with a 6 M guanidine - HCl -0.4 M Tris-HCl buffer (pH 8.5) to remove excessive reagent 15 and followed by reverse phase HPLC using a reverse phase column (Cosmasil 10C18-300, 4.6 mm I.D. X 100 mm, Nakalai Tesque Inc.). A fraction having both absorption at 280 nm derived from tryptophane and 254 nm derived from pyridine by UV detector was collected.

20 Following this step, the trypsin digestion was performed by the same method as that of Example 1 (3) to separate digested fragments.

Finally, the position of respective fragments in the primary structure of mature MP52 was determined by the amino acid composition analysis as same as that of Example 1 (3).

Using the reverse phase HPLC, a comparison of elution times of respective trypsin fragments of unmodified mature human MP52 and methionine alkylated mature human MP52 of which position in the primary sequences was determined by the amino acid composition analysis was performed. Trypsin fragments of mature human MP52 containing a methionine residue were three kinds corresponding to: the amino acid position from 29th to 56th (29-56; containing 30th methionine) shown in SEQ ID N° 1, the amino acid position from 57th to 88th (57-88; containing 71st and 74th methionines) in SEQ ID N° 1, and the amino acid position from 107th to 119th (107-119; containing 111th methionine) in SEQ ID N° 1. The elution time of respective fragments derived

19

from unmodified mature human MP52 were around 77 min, 58 min, and 36 min in order. In comparison, fragments derived from methionine alkylated mature human MP52 (SEQ ID N° 6) were around 74 min, 42 min, and 30 min in order, showing the earlier elution than that of the fragments of unmodified mature human MP52.

On the other hand, the elution time of other fragments without containing methionine was the same between unmodified and methionine alkylated mature human MP52.

In the amino acid composition analysis, only the number of methionine residues derived from the methionine alkylated mature human MP52 was different from the theoretical values. The N-terminal sequence of these fragments were analyzed by using a sequencer (model 476A; Applied Biosystems) and it is confirmed that the alkylation occurred to methionine residues specifically.

Example 3 Preparation of mature human MP52 of which tryptophane was allylsulphenylated

(1) Allylsulphenylation of tryptophane residues of mature 20 human MP52 by p-nitrophenylchloride

Twenty-fold molar ratio of p-nitrophenylchloride, which dissolved in 100% acetic acid, was added to the unmodified mature human MP52 (2 mg/ml dissolved in 50% acetic acid) and incubated at room temperature for 1 hour.

25 (2) Separation of allylsulphenylated mature human MP52

Unmodified mature human MP52 and allylsulphenylated
mature human MP52 having the amino acid sequence of SEQ ID

N° 7 were separated on the basis of a difference in the
retention time of column on the reverse phase HPLC. The

30 elution time of allylsulphenylated mature human MP52 delays
in comparison with unmodified mature human MP52 on the
reverse phase HPLC, which is due to increase in hydrophobic
property by allyl sulphenylation. The conditions of
separation are as follows; a column employed, a flow rate,
35 and a pump for HPLC were the same as those of Example 1 (2),
and column temperature and wavelengths for detection were
40°C, and 214 nm and 365 nm, respectively. For solvent,

water containing 0.05% TFA as solution A and acetonitrile

20

containing 0.05% TFA as solution B were used for elution.

The elutions of proteins were performed by a linear gradient of solution B from 25% to 60% for 35 minutes after maintaining for five minutes with 25% of solution B. Under the conditions, unmodified mature human MP52 was eluted around 22 min, and in comparison, tryptophane allylsulphenylated mature human MP52 was eluted around 26 min. The difference of elution times between these proteins allows easy separation of them.

10 (3) Determination of allylsulphenylation of tryptophane

After reduction of disulfide bond by the same method
described in Example 1, unmodified mature human MP52 of which
cysteine residues were alkylated (S-carboxymethylated) and
tryptophane allylsulphenylated mature human MP52 were
15 digested by trypsin, and fragments of the digest were
separated by C18 reverse phase column. Amino acid positions
of the fragments were determined by the amino acid
composition analysis.

The trypsin fragment of mature human MP52 containing

tryptophane residues was that corresponding to the position

from 29th to 56th positions (29-56; containing 32nd and 35th

positions of tryptophane) alone shown in SEQ ID N° 1. Only

the elution time (around 95 min) of a fragment containing

tryptophane residue, which is derived from allylsulphenylated

mature human MP52 (SEQ ID N° 7) delayed in comparison with

the elution time (around 84 min) of a fragment derived from

the unmodified mature human MP52. On the other hand, the

elution time of other fragments without containing

tryptophane showed no change. N-terminal sequence analysis

showed difference only in tryptophane residue between

unmodified and modified mature human MP52.

Example 4 Test of inhibition of bone morphogenetic activity

BMP-2 antagonistic activity of mature protein of this
invention was tested by adding the protein to culture media
of MC3T3-E1 cells which are a murine carvarial clonal cell
line established by Dr. Kodama et al. and have osteoblastlike properties, or C3H10T ½ cells which are a mesenchymal
and multipotent cell line to differentiate to osteoblast,

21

chondrocyte, myocyte, fat cell, according to a culture condition. These cell lines were cultured according to the method described by Dr. Takuwa et al. For the MC3T3-E1 cells, 5 X 10<sup>3</sup> cells per cm<sup>2</sup> were inoculated and cultured in 5 α-MEM medium containing 10% fetal bovine serum for three days. After washing cells with the α-MEM medium, the medium was replaced into α-MEM medium containing 0.3% bovine serum albumin. To this medium, the mature protein of the invention with various concentrations and mature rh-BMP-2 (recombinant human BMP-2) were added and then cultured for three days (post-culture). ALPase activity in the cells was measured by colorimetric analysis using p-nitrophenylphosphate as a substrate.

The culture conditions of C3H10T ½ cells were the same 15 as MC3T3-E1 cells except that the medium used for the preculture was BME culture medium containing 10% fetal bovine serum and the medium used for the post-culture was BME culture medium containing 2% fetal bovine serum.

Fig. 1 shows the result of ALPase activity by methionine 20 alkylated mature human MP52 in comparison with that by unmodified mature human MP52 in MC3T3-E1 cells. proteins were obtained from the difference in the retention time on the reverse phase HPLC in Example 2. In the figure, the solid line with solid circles represents unmodified 25 mature human MP52, the solid line with solid squares represents one-methionine alkylated mature human MP52, the broken line with solid triangles represents two-methionine alkylated mature human MP52, the solid line with open circles represents three-methionine alkylated mature human MP52, the 30 dotted line with open triangles represents four-methionine alkylated mature human MP52, respectively. The solid circles shown in of the Y-axis present activity of cells without treated by any reagent. As shown in Fig. 1, ALPase inducing activity in MC3T3-E1 cells decreased depending on the 35 progress of alkylation of methionine.

Fig. 2 shows the antagonistic activity of methionine oxidized mature human MP52 obtained in example 1 and tryptophane allylsulphenylated mature human MP52 obtained in

22

example 3 in two different cell lines. Fig. 2 (A) represents an antagonistic activity against mature rh-BMP-2 in C3H10T % cells. Fig. 2 (B) shows an antagonistic activity against mature human MP52 in MC3T3-El cells. In both figures, the solid line with solid circles represents tryptophane allylsulphenylated mature human MP52 and the dotted line with open circles represents methionine oxidized mature human MP52, respectively. In Fig. 2(A), the solid square shows ALPase activity induced by 300 ng/ml of mature rh-BMP-2 alone in C3H10T % cell. In Fig. 2 (B), the solid square shows ALPase activity induced by 600 ng/ml of unmodified mature human MP52 alone in MC3T3-El cells. Open squares represent the activity in the experiments without any reagent.

As shown in Fig. 2(A), 300 ng/ml of mature rh-BMP-2

15 promoted ALPase activity in C3H10T ½ cell about 40 times higher than the control group. The modified mature human MP52 of this invention inhibited the ALPase activity from 1 equivalent- to 20 equivalent-molar in a dose-dependent manner. Moreover, as shown in Fig. 2(B), 600 ng/ml of the 20 unmodified mature human MP52 promoted ALPase activity in MC3T3-El cells about 3 times higher than the control group. The amino acid modified mature human MP52 of this invention inhibited the ALPase activity from 1 equivalent- to 10 equivalent-molar in a dose-dependent manner.

WO 00/21998

PCT/IB99/01621

23

## Sequence Listing Free Text

<210> 1

<223> Mature MP52

5

<210> 2

<223> Mature BMP-2

<210> 3

10 <223> Mature BMP-4

<210> 4

<223> Mature BMP-7

15 <210> 5

<223> Mature MP52 protein. Note: 30th, 71st, 74th and 111th Met are modified to Met sulfoxide.

<210> 6

20 <223> Mature MP52 protein. Note : 30th and/or 71st and/or 74th and/or 111th Met are modified to s-carboxymethyl Met.

<210> 7

25 <223> Mature MP52 protein. Note: 32nd and 35th Trp are modified to allylsulphenyl Trp.

PCT/IB99/01621

24

#### What is claimed is:

- A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.
- 10 2. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an oxidization reaction.
- The mature protein according to claim 2 in which four methionine residues are oxidized and having the amino acid
   sequence of SEQ ID N° 5.
  - 4. The mature protein according to claim 1, wherein the chemical modification for said methionine residue is an alkylation reaction.
- 5. The mature protein according to claim 4 wherein the alkylation reaction is S-carboxymethylation in which at least one methionine residue is S-carboxymethylated and having the amino acid sequence of SEQ ID N° 6.
- 6. The mature protein according to claim 1, wherein the chemical modification for said tryptophane residue is an 25 allylsulphenylation reaction.
  - 7. The mature protein according to claim 6 in which two tryptophane residues are allylsulphenylated and having the amino acid sequence of SEQ ID  $N^{\circ}$  7.
- 8. The mature protein according to claims 1 to 7, wherein 30 said mature human MP52 is a dimer protein.
  - 9. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID N $^{\circ}$  2), mature
- 35 human BMP-4 (SEQ ID N° 3) or mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.

25

- 10. A mature protein having an antagonistic activity against bone morphogenetic proteins, obtained by replacing at least one amino acid residue of three hydrophobic amino acid residues, among said hydrophobic amino acid residues relating to a receptor binding site in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), or mature human BMP-7 (SEQ ID N° 4), which are located in positions corresponding to those of methionine residues located in 30th, 71st, and 74th positions of the amino acid sequence of mature human MP52 (SEQ ID N° 1) with a hydrophilic amino acid residue or a polar amino acid residue.

  11. The mature protein according to claim 9 or 10, wherein said mature human BMP-2, mature human BMP-4, or mature human
- 15 12. An agent for therapy and/or prevention of ectopic ossification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.

BMP-7 is a dimer protein.

13. An agent for therapy and/or prevention of metabolic 20 diseases with calcification, containing a mature protein according to any one of claims 1 to 11 as an effective ingredient showing an antagonistic activity against a bone morphogenetic protein.



## PCT

# WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau

# INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup>:
C07K 14/51, A61K 38/18, A61P 19/00, 21/00, 9/10

(11) International Publication Number:

WO 00/21998

A1 (4

(43) International Publication Date:

20 April 2000 (20 04.00)

(21) International Application Number:

PCT/IB99/01621

(22) International Filing Date:

4 October 1999 (04.10.99)

(30) Priority Data:

10/288103

9 October 1998 (09.10.98)

JP

(71) Applicant (for all designated States except US): HOECHST MARION ROUSSEL [FR/FR]; 1, terrasse Bellini, F-92800 Puteaux (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): KATSUURA, Mieko [JP/JP]; 2-14-2-106, Sakae-cho, Higashimurayama-shi, Tokyo 189-0013 (JP). KIMURA, Michio [JP/JP]; 9-8-304, Tsurugadai, Chigasaki-shi, Kanagawa 253-0003 (JP).

(74) Agent: VIEILLEFOSSE, Jean, Claude; Hoechst Marion Roussel, 102, route de Noisy, F-93235 Romainville Cedex (FR).

(81) Designated States: AE, AL, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, TZ, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

#### Published

With international search report.

(54) Title: BONE MORPHOGENETIC PROTEIN ANTAGONIST BASED ON THE MATURE PROTEIN

### (57) Abstract

The purpose is to provide a mature protein having an antagonistic activity against bone morphogenetic proteins. The mature protein having an antagonistic activity against bone morphogenetic proteins is obtained by converting at least one residue among methionine residues or tryptophane residues existing in the amino acid sequence of mature human MP52 (SEQ ID N° 1) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue. The chemical modification for said methionine residue is performed by an oxidization reaction or an alkylation reaction. The chemical modification for said tryptophane residue is performed by an allylsulphenylation reaction. Or a mature protein having an antagonistic activity against bone morphogenetic proteins is obtained by converting at least one residue of tryptophane residues existing in the amino acid sequences of mature human BMP-2 (SEQ ID N° 2), mature human BMP-4 (SEQ ID N° 3), and mature human BMP-7 (SEQ ID N° 4) to a hydrophilic residue by chemical modification, or replacing said residues with a hydrophilic amino acid residue or a polar amino acid residue.

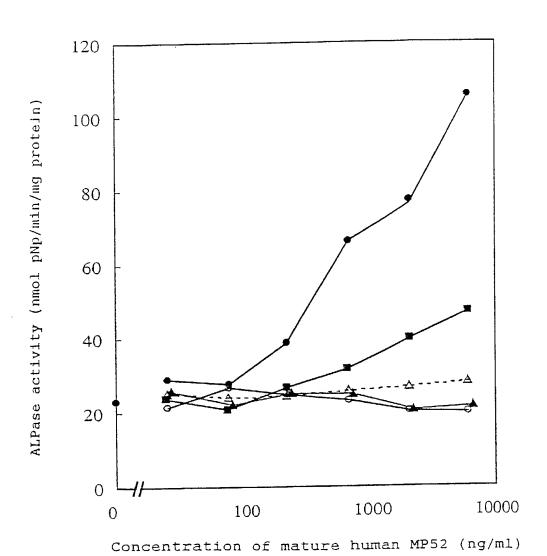
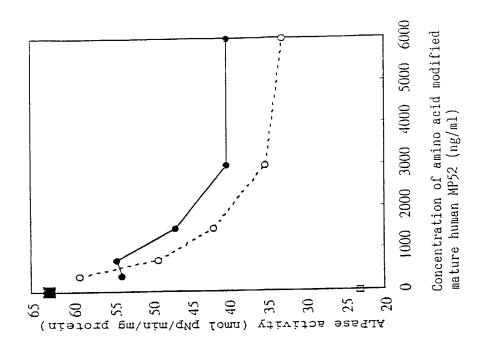


FIGURE 1

WO 00/21998

PCT/IB99/01621

2/2



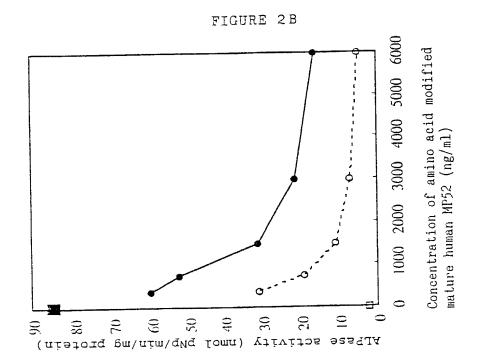


FIGURE 2A

PCT Applicant's Guide - Volume II - National Chapter - US PTO/SUCT (8:24)
Approved for use through 9,0098 CAB 0651,0032
Patentiand Trademark Office U.S. SEPARTMENT OF COMMERCE or 1995, no persons are required to respond to a collection of information unless 4 contains a vaid CMB control number Attorney Docket Number, 447.001 KATSUURA et al First Named Inventor **DECLARATION FOR** COMPLETE IF KNOWN UTILITY OR DESIGN PCT/IB99/01621 Application Number PATENT APPLICATION October 4, 1999 Filing Date Group Art Unit Declaration OR Declaration Submitted after Submitted Initial Filing with Initial Filing Examiner Name As a below named inventor, I hereby declare that: My rosidence, post office address, and citizenship are as stated below I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are leaded below) of the subject matter which is claimed and for which a patent is sought on the invention entitled; BONE MORPHOGENETIC PROTEIN ANTAGONIST BASED ON THE MATURE PROTEIN (Take of the Invention) OR as United States Application Number or PCT International XXwas filed on (MM/DOYYYY) 10/04/99 PCT/IB99/0162 1 and was amended on (MINDOMYY) (diapplicable) I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above Lacknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56 I hereby etaim foreign priority benefits under Title 35, United States Code §119 (2)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365 (a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is daimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYY)	Priority Not Claimed	Certified Co	py Attached?	
10/288103	Japan	10/09/98	סטכ			
PCT/IB99/0162	1 PCT	10/04/99				
Additional foreign applicati	on numbers are Ested on a supplemental p	riority sheet attached he	ereta:			

I hereby claim the benefit under Title 35,	United States Code § 119(e) of any United State	s provisional application(s) listed below
Application Number(s)	Filing Date (MM/DD/YYYY)	C Addisont on the

Application Number(s)	Filing Date (MM/DD/YYYY)	Additional provisional application						
•	·	numbers are listed on a supplemental priority sheet attached hereto.						
•		_						

Annex US III, page 2 MM PCT Appelan

ant's Guide - Volume II - National Chapter - US

Under the Paperwork Reduction Act of 1995, no genuins are required to respond to a collection of information unless it contains a valid CMB control number

# DECLARATION

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, incolar as the subject matter of each of the chims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which acknowledge the duty to disclose information which is material to patentability the paragraph of the prior application and the national or PCT international Ring date of this application.

U.S. Parent Application Number	Po	CT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parer	nt Patent Number if applicable)
-					
		Ŷ			
Additional U.S. or PCT international	l application re	umbers are listed on a	supplemental priority sheet atta	iched herelo	- i - i - th - Datast
As a named inventor, I hereby appoint to	ha laflowing re	gistered practioner(s	) to prosecute this application as	nd to transact all b	usness in the Patent
and Trademark Office connected therev	ve.i.,	Registration Number	Name		Registration Number
ierman, Muserlia Lucas	n and	18,818			
ordan E. Bierman Charles A. Muserl Donald C. Lucas	ian	18,629 19,683 31,275	-		
Additional registered practitions	er(s) named	on a supplemental :	sheet attached hereto.		
Direct all correspondence to:					
Name   Charles A.	Muser	Tian			
Address Bierman, M	userli	an and Lu	īcās		
Address 600 Third					
Mou Vonk			State NY	ZIP	10016
II S A	Te	lephone (212	661-8000	Fax (21	
Country U, J. H.  I hereby declare that all statements made be true; and further that these statement imprisonment, or both, under Section 1 the application or any patent issued the	nts were made 100 of Title 180	y own knowledge are t with the knowledge to tol the United States	Code and that such willul false	statements may	jeopardize the validity of
Name of Sole or First Invento			A petition has been f	iled for this unsi	gned inventor
Given Mieko			amily Katsuura		e.g. Jr.
	0 1	1. Thursday	-	Date A	pril 25,200/
Inventor's Signature Muel	20 K	avmuia			
Residence: City Tokyo	794	State Co	Japan ·		Citizenship Japan
Signature Much	794	State Co	- o apan		
Residence: City Tokyo	794	State Co	- o apan		

US

PCT Applicant's Guide - Volume II - National Chapter - US

Annex US III, page 3

PTOCEST: (8.90)
Approved for use through 9/30/36 CMS 0651-0337
Patent and Trademark Office U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless 2 contains a valid CME control number ADDITIONAL INVENTOR(S) DECLARATION Supplemental Sheet A petition has been filed for this unsigned inventor Name of Additional Joint Inventor, if any: Middle Michie Initial Name inventor's Date Signature Country Residence: Japan City Post Office Address Tsurugadai, Chigasaki-Shi, Kanagawa 253-0003 9-8-304, Japan Post Office Address City Japan Kanagawa A petition has been filed for this unsigned inventor Name of Additional Joint Inventor, if any: Suma Family Middle Given Inidal Name Name Date Inventor's Signature Curcustia Residence: Country State City Post Office Address Post Office Address State Zip Country A petition has been filed for this unsigned inventor Name of Additional Joint Inventor, if any: Suffix Middle Family Given Name Inventor's Date Signature Residence: Citizenship Country City Post Office Address 4 Post Office Address City Country A petition has been filed for this unsigned inventor Name of Additional Joint Inventor, if any: Middle Family Given Name inventor's Oate Signature Residence: Citizenstilp State Country City Post Office Address Post Office Address

Country

Ζiρ Additional inventors are being named on supplemental sheet(s) attached hereto

## JC03'Rec'd PCT/PTO 2 8 MAR 2001 PCT/IB99/01621

WO 00/21998

1

## SEQUENCE LISTING

<110> Hoechst Marion Roussel

<120> MATURE PROTEIN HAVING ANTAGONIST ACTIVITY AGAINST BONE MORPHOGENETIC PROTEIN.

<130> JH98K011 PCT SEQUENCES IN ENGLISH

<140>

<141>

<150> 10-288103

<151> 1998-10-09

<160> 7

<170> PatentIn Ver. 2.1

<210> 1

<211> 119

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(119)

<223> Mature MP52

<300>

<301> MAKISHIMA, Fusoa
 TAKAMATSU, Hiroyuki
 MIKI, Hideo
 KAWAI, Shinji
 KIMURA, Michio
 MATSUMOTO, Tomoaki
 KATSUURA, Mieko

ENOMOTO, Koichi

WO 00/21998

PCT/IB99/01621

2

SATOH, Yusuke

<302> Novel protein and process for producing the same.

<310> WO 95/33215

<312> 1996-10-24

<313> 1 TO 119

<400> 1

Pro Ser Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala 1 5 10 15

Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp
20 25 30

Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu 35 40 45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
50 55 60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro 65 70 75 80

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe 85 90 95

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val

100 105 110

Val Glu Ser Cys Gly Cys Arg 115

<210> 2

<211> 114

<212> PRT

<213> Human

3

<220>

<221> CHAIN

<222> (1) .. (114)

<223> Mature BMP-2

<300>

<301> WANG, Elizabeth A.

WOZNEY, John M.

ROSEN, Vicki A.

<302> Novel osteoinductive compositions.

<310> WO 88/00205

<312> 1988-01-14

<313> 1 TO 114

<400> 2

Gln Ala Lys His Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg

1 5 10 15

His Pro Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile
20 25 30

Val Ala Pro Pro Gly Tyr His Ala Phe Tyr Cys His Gly Glu Cys Pro 35 40 45

Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln
50 55 60

Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys Ala Cys Cys Val
65 70 75 80

Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu
85 90 95

Lys Val Val Leu Lys Asn Tyr Gln Asp Met Val Val Glu Gly Cys Gly
100 105 110

4

<210> 3

<211> 116

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(116)

<223> Mature BMP-4

<300>

<301> WOZNEY, John M.

ROSEN, Vicki

CELESTE, Anthony J.

MITSOCK, Lisa M.

WHITTERS, Matthew J.

KRIZ, Ronald W.

HEWICK, Rodney M.

WANG, Elizabeth A.

<302> Novel regulators of bone formation : molecular clones and activities.

<303> Science

<304> 242

<305> 4885

<306> 1528-1534

<307> 1988-12-16

<308> Genbank/M22490

<313> 1 TO 116

Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys 15 10 5

Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp 30 20 25

Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp 45

WO 00/21998

PCT/IB99/01621

5

Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His Ala Ile 50 55 60

Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile Pro Lys Ala Cys
65 70 75 80

Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu 85 90 95

Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met Val Val Glu Gly
100 105 110

Cys Gly Cys Arg

<210> 4

<211> 139

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(139)

<223> Mature BMP-7

<300>

<301> OZKAYNAK, Engin

RUEGER, David C.

DRIER, Eric A.

CORBETT, Clare

RIDGE, Richard J.

SAMPATH, Kuber T.

OPPERMANN, Hermann

<302> OP-1 cDNA encodes an osteogenic protein in the TGF-beta family.

6

<303> EMBO J.

<304> 9

<305> 7

<306> 2085-2093

<307> 1990

<308> EMBL data library/X51801

<313> 1 TO 139

<400> 4

Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro Lys

1 5 10 15

Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser Ser 20 25 30

Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe Arg

35 40 45

Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala Ala 50 55 60

Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met Asn 65 70 75 80

Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn Pro 85 90 95

Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala Ile
100 105 110

Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys Tyr 115 120 125

Arg Asn Met Val Val Arg Ala Cys Gly Cys His 130 135

7

<210> 5

<211> 119

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(119)

<223> Mature MP52 protein. Note: 30th, 71st, 74th and 111th Met are modified to Met sulfoxide.

<400> 5

Pro Ser Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala

1 5 10 15

Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp
20 25 30

Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu 35 40 45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
50 55 60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro 65 70 75 80

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe 85 90 95

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val
100 105 110

Val Glu Ser Cys Gly Cys Arg

8

<210> 6

<211> 119

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(119)

<223> Mature MP52 protein. Note : 30th and/or 71st and/or 74th and/or 111th Met are modified to s-carboxymethyl Met.

<400> 6

Pro Ser Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala

1 10 15

Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp
20 25 30

Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu 35 40 45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
50 55 60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro 65 70 75 80

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe 85 90 95

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val
100 105 110

Val Glu Ser Cys Gly Cys Arg

9

<210> 7

<211> 119

<212> PRT

<213> Human

<220>

<221> CHAIN

<222> (1)..(119)

<223> Mature MP52 protein. Note : 32nd and 35th Trp are modified to allylsulphenyl Trp.

<400> 7

Pro Ser Ala Thr Arg Gln Gly Lys Arg Pro Ser Lys Asn Leu Lys Ala
1 5 10 15

Arg Cys Ser Arg Lys Ala Leu His Val Asn Phe Lys Asp Met Gly Trp

Asp Asp Trp Ile Ile Ala Pro Leu Glu Tyr Glu Ala Phe His Cys Glu 35 40 45

Gly Leu Cys Glu Phe Pro Leu Arg Ser His Leu Glu Pro Thr Asn His
50 55 60

Ala Val Ile Gln Thr Leu Met Asn Ser Met Asp Pro Glu Ser Thr Pro 65 70 75 80

Pro Thr Cys Cys Val Pro Thr Arg Leu Ser Pro Ile Ser Ile Leu Phe 85 90 95

Ile Asp Ser Ala Asn Asn Val Val Tyr Lys Gln Tyr Glu Asp Met Val
100 105 110

Val Glu Ser Cys Gly Cys Arg